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(57) Abstract

A method and a kit for the diagnosis of equine toxicoinfectious clostridiosis which comprises detecting the presence of an antibody to a *C. botulinum* antigen or a phenotype thereof and/or an antibody to a clostridial toxin in a biological sample. The method is preferably used for the detection of equine grass sickness.

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DIAGNOSIS OF TOXICOINFECTIOUS CLOSTRIDIOSIS

The invention relates to the diagnosis of equine toxicoinfectious clostridiosis, particularly to the diagnosis of grass sickness (also known as equine dysautonomia).

Equine Grass Sickness (EGS), also known as Mal Seco in Argentina, is an unpredictable disease which characteristically affects younger individual horses in successive generations grazing contaminated pastures and over a time-scale measured in decades. EGS is one manifestation of toxicoinfectious clostridiosis. Clinical EGS cases may be seen as manifestations of a complex of equine gastro-intestinal and/or neurologic disorders.

The present inventor has found that horses grazing in endemic EGS areas are invariably seroconverted to a Clostridium novyi phenotype. The evidence of sporadic disease in such populations demonstrates the limitations of natural immunoprotection, which is also dependent upon effective operation of the immune response at the individual horse level. Clostridial toxicoinfectious disease may present a variety of non-specific gastro-intestinal and/or neurologic signs which are not pathognomic, especially in older animals, thus exacerbating the problem of diagnosis of EGS.

Previously, diagnosis of EGS could only be confirmed by ileal biopsy (laparotomy) or at autopsy.

In 1924 Tocher and his colleagues presented evidence

from which they concluded that equine Grass Sickness (EGS) is a form of botulism. The contention was immediately discredited on the basis of inadequate microbiological corroboration and for its failure to explain notable clinical features of EGS which are dominated by dysautonomia (i.e. disturbance of the autonomic nervous system) and which are patently different from those of classical equine botulism or forage poisoning. The botulinum toxin theory as it related to EGS was further undermined by apparent failure of a prototype vaccine (which was prepared by combining Clostridium botulinum Type A toxin with homologous antitoxin, in vitro) to control disease in susceptible populations of horses.

In the intervening years research interest has focused on the neuronal lesions of EGS which were believed to be the essential feature of the disease and to be evidence of a neurotoxic influence but apparently irreconcilable with botulinum toxin involvement. Furthermore, in a recent review Pollin and Griffiths concluded that there were no literature references to infectious agents or toxins capable or likely to be capable of causing the type and distribution of neuronopathy which confirms the autopsy (or biopsy) diagnosis of EGS.

Studies which led to the present invention were based on two fundamental assumptions. Firstly that EGS is a toxicoinfectious disease process which depends upon prior colonization of the bowel (or an intercurrent disease

lesion) with toxin-producing clostridia and secondly, that the strain of Clostridium botulinum responsible for EGS invariably produces two neuroactive toxins. Thus the concerted toxic insult could account for the dysautonomia and for the classical gastro-enteric and neurologic manifestations of EGS so providing a satisfactory explanation for the most controversial aspect of the disputed botulinum theory.

It is known that the majority of Group III strains of Clostridium botulinum (Types C and D) produce a variable yield of secondary toxins in association with their type specific neurotoxins (C_1 and D_1). Clostridium botulinum Type C_2 binary toxin was recognized to be of particular relevance in the context of EGS in view of its well documented role as a potent ADP ribosyltransferase: thus C_2 toxin has the capacity to activate a broad spectrum of cell membrane receptors which have been characterised by in vitro studies and in laboratory animal models. C. botulinum types C and D are the only serotypes which have the capacity to elaborate the combination of neuroactive and enterotoxic components necessary to account for the dramatic clinical signs of EGS.

A number of bacterial protein toxins interfere with the processes of cellular communication, or signal transduction, by acting as freelance ADP ribosyltransferases. Additional ribose groups are added to the ADP molecule to form a polyribose complex which cannot

be phosphorylated to ATP.

Individual ADP ribosyltransferases have specific substrates which are activated to disrupt membrane function operating systems and their coupled intercellular processes: in the case of botulinum C_2 toxin the substrate is monomeric G-actin, a guanylate protein, which forms the cytoskeletal structure of all secretory and motile cells derived from the embryonic neural crest.

The precisely targeted consequences of botulinum C_2 toxin-induced ADP ribosylation are considered to be responsible for the neuronal lesions associated with EGS, and for their pattern of peripheral distribution from an enteric toxicoinfectious focus by the process of retrograde axonal transmission. Studies carried out in vitro have demonstrated that neuronal exposure to C_2 binary toxin leads to uncontrolled neurochemical discharge and similar exposure inhibits the activity of motile cells. These events are accompanied by ultrastructural changes, defined as chromatolysis, which indicate extreme physiological stress: more prolonged or more concentrated exposure to C_2 toxin leads to cell lysis. These are the neuronal lesions associated with equine dysautonomia. However, prior to the present implication of C. botulinum Type C_1 and C_2 toxins in the neurotoxicology of EGS, there had been no previous recorded indictment of the two toxins in any human or animal disease process.

Type C organisms present in carrion are responsible

for most epizootic botulism but such outbreaks (associated with the ingestion of preformed toxin) which may involve horses, or other species, are not associated with any clinical evidence of C_2 toxin involvement.

Type C_1 neurotoxin inhibits the release of acetyl choline from its storage vesicles by a specific zinc dependent cleavage of syntaxin, a neuronal endopeptide.

The present inventor has demonstrated a relationship between a primitive organism and a highly specific G-protein mediated signal transduction system, the phosphotidylinositol cascade, which mediates all calcium-sensitive processes such as secretion and chemotaxis.

Toxicoinfectious manifestations of botulism are frequently encountered on lower animals: significantly, in the context of EGS, all avian infections (which predominantly involve Type C organisms) are toxicoinfectious reflecting the evolutionary significance of this intimate host-parasite relationship. However, it was not until 1976 that a human infant disease was associated with the spontaneous elaboration of botulinum toxins <u>in vivo</u>: so called infant botulism is a well recognised cause of morbidity and mortality in the U.S. where it results from ingestion of botulinum spores (Types A and B) predominantly by susceptible infants in the 3-6 month age range.

Classical toxicoinfectious botulism of horses (the Shaker Foal Syndrome) which is prevalent in Kentucky,

elsewhere in the eastern US and which occurs in Australia is associated with C. botulinum Type B. However, this condition is more akin to human wound botulism since it can only be reproduced in the presence of pre-existing anaerobic lesions which may be clinically insignificant but which nevertheless provide a focus for localization of the toxigenic agent.

As the name suggests Grass Sickness is almost invariably associated with access to grazing and the seasonal pattern of disease is generally assumed to result from exposure to a transient toxic factor in herbage. However, the present inventor has postulated that contagion results from clostridial spores in soil ingested during grazing: such spores would be expected to vegetate in the bowel to initiate a specific immune response which will signify the carrier state.

Horses in enzootic EGS areas develop antibodies to a phenotype of Clostridium botulinum (Group III), demonstrating the toxicoinfectious nature of the disease process. A meaningful pattern of serological (i.e. antibody related) responses indicates the degree of previous exposure and provides a measure of susceptibility of disease.

However, the serodiagnostic approach is complicated by the fact that all horses harbour vast enteric populations of non-pathogenic clostridia. Therefore testing methods leading to the present invention took

account of between-group and within-group comparisons of clostridial seroconversion in clinically normal horses and those in specific disease categories as demonstrated in the Examples set out below.

The invention provides a method for the diagnosis of equine toxicoinfectious clostridiosis which comprises detecting the presence of an antibody to a C. botulinum antigen or a phenotype thereof or an antibody to a clostridial toxin in a biological sample. The method of the invention is useful for the diagnosis of manifestations of equine toxicoinfectious clostridiosis such as anterior enteritis or colitis X which are associated with toxinogenic strains of C. perfringens.

More preferably, the invention relates to a method for diagnosis of equine grass sickness which comprises detecting the presence of an antibody to C. botulinum Group III antigen or a phenotype thereof, and also detecting the presence of an antibody to a botulinum type C toxin.

By phenotype is meant herein a clostridial-derived antigen provoking a similar immunological reaction to types of C. botulinum. In the case of C. botulinum Group III (types C and D), the phenotype is preferably derived from Clostridium novyi Type A. The method of the invention is preferably an ELISA method.

The invention further provides a kit for diagnosis of equine toxicoinfectious clostridiosis, which comprises means for detecting an antibody to a C. botulinum antigen

or a phenotype thereof and/or means for detecting an antibody to a clostridial toxin. The kit may be adapted for field use.

Preferably, the invention relates to a kit for diagnosis of equine grass sickness, which comprises means for detecting the presence of an antibody to C. botulinum Group III antigen or a phenotype thereof and means for detecting an antibody to a botulinum type C toxin.

The test method according to the invention permits identification of apparently normal horses which have developed antibody to cell wall antigens of the indicator organism Clostridium novyi; individual clinical cases of suspected EGS can be identified as such by the detection of antibodies (antitoxins) to the EGS-specific neurotoxin. Hitherto, confirmation of EGS diagnosis *in vivo* required stressful and costly laparotomy and ileal biopsy in equine hospitalisation facilities.

The demonstration of a rising serological (i.e. antibody) response to C. novyi cell surface antigens in parallel with a decreasing response to botulinum C_1 toxin in EGS affected horses typifies the toxicoinfectious nature of the disease. The bowel has been identified as the site of toxin elaboration at least in classical manifestations of EGS.

Monitoring of the results of the test of the invention has confirmed that many "carrier" horses are exposed to the toxicoinfectious agent of EGS and that

evidence of such exposure, at the individual horse level, can be determined by titration of the antibody response to C. novyi, the indicator phenotype for C. botulinum Types C/D. The fact that very few carrier horses succomb to classical or atypical EGS attests to the general effectiveness of immunosuppressive mechanisms and/or detoxification processes. In suspect clinical cases evidence of a serological response (i.e. antitoxin titre) to a primary EGS toxin, now identified as botulinum Type C_1 neurotoxin, denotes failure of the immune or protective response. These tests can be used separately to identify infected animals, or preferably, will be used in combination to provide a reliable method for the diagnosis of EGS.

Cell surface preparations of Clostridium sporogenes, deposited at the National Collection of Type Cultures at Colindale as NCTC 8594, Clostridium perfringens deposited as NCTC 8237 and Clostridium novyi deposited as NCTC 538 provide antigens which are phenotypically representative of Clostridium botulinum Group I (types A, proteolytic B and F), Group II (types non-proteolytic B and E) and Group III (types C and D) respectively. The inventor has found that Group III of Clostridium botulinum, and the phenotypically representative antigen derived from C. novyi are mainly responsible for toxicoinfectious clostridiosis.

Biological samples, preferably serum samples are tested according to the method of the invention by an

adaptation of a standard ELISA technique (enzyme-linked immunosorbant assay). Suitably, a non-competitive ELISA method is used according to the invention.

Enzyme-linked immunosorbent assay (ELISA)

The diagnostic value of an ELISA technique is dependent upon purity of antigens and especially so for the characterization of anaerobic infections where the antigenic structures are uncertain and variable.

Hence in the present application screening versatility of the test procedure may be provided by strategic use of somatic antigens derived from appropriate clostridial phenotypes viz. Clostridium novyi, C. sporogenes and C. perfringens which combine to indicate responses to a broad spectrum of toxinogenic clostridia including all known strains of Clostridium botulinum. Thereafter specific "diagnostic" analysis of the serological response is ensured by the use of purified toxin antigens precisely to categorize individual antitoxin components of the host reaction to toxicoinfectious discharge.

An ELISA protocol, suitable for use in the diagnostic method of the invention is as follows:

1. Dilutions of antigen are made in coating buffer (0.05M sodium carbonate buffer pH 9.6 containing 0.02% sodium azide) and 100 µl volumes, containing 10-100 µg antigen are added to wells of microtitre plates. Plates are covered and incubated at 37°C for 4 h and then at 4°C

overnight.

2. The plates are washed three or four times with 0.9% Na Cl containing 0.05% Tween 20.

3. Dilutions of antibody in antibody conjugate buffer are made (0.05M phosphate buffer, pH 7.4, containing 0.85% Na Cl, 0.05% Tween 20 and 0.02% sodium azide) and added to wells. The wells are incubated for up to 4 h at room temperature.

4. Washing Step 2 is repeated.

5. 100µl volumes of suitably diluted anti first species antibody - enzyme conjugate (doubling dilutions from 1 in 500) are added to the wells which are incubated overnight at room temperature (enzyme conjugate used horseradish peroxidase).

6. Washing Step 2 is repeated.

7. Diluted enzyme substrate is added and the wells are incubated at room temperature for 1 h. Results are read spectrophotometrically.

The sample containing the Group III antigen or analogue thereof may be a biological fluid or tissue sample, and is preferably serum.

The diagnostic method according to the invention provides a rapid, non-invasive and conclusive method for confirming the diagnosis of equine dysautonomia, otherwise known as grass sickness. The method allows for the differential diagnosis of clostridial involvement in non-specific gastro intestinal and/or neurologic equine

disorders.

As a further feature, the method of the invention may be used as a facility for monitoring responses to treatment of clinical toxicoinfectious clostridiosis, including Grass Sickness, in individual horses. Host responses to individual clostridial toxins involved in the toxinology of equine toxicoinfectious clostridiosis may also be identified and quantified by performing the method of the invention and interpreting the results obtained.

The diagnostic method of the invention may also be used as the basis of a technique for the parallel assessment of bacterial virulence factors or toxins which combine to determine the pathogenesis of field strains of toxinogenic clostridia.

Identification and quantification of host responses to individual clostridial toxins together with the parallel assessment of bacterial virulence factors or toxins responsible for the pathogenesis of specific toxinogenic clostridia are prerequisites for the epidemiological study of equine toxicoinfectious disease.

The invention is further illustrated by the following Examples. In the Examples, four categories of horses showing clinical signs were identified. The groups are as follows:

Group I - Acute Grass Sickness (AGS)

Group II - Chronic Grass Sickness (CGS)

Group III - Clinical gastroenteric (GE) including acute

abdominal crises.

Group IV - Clinical miscellaneous including specimens submitted from a broad spectrum of diagnosed cases or with key presenting signs.

Example 1 - Determination of indicator antigens

A group of 34 clinical specimens was tested by the (IgG) ELISA technique at two serum dilutions i.e. 1/25 and 1/50. Each specimen was tested in duplicate and ELISA OD values were presented as a mean of the two results.

Table 1 shows the mean OD values (with ranges and variance) for the four clinical groups, against the three indicator antigens.

TABLE 1

ELISA OD VALUES AT 1/50 SERUM DILUTION

Antigen		I AGS	II CGS	III GE	IV nonGE
	n	9	23	12	25
C. novyi	mean	0.302	0.634	0.677	0.399
	range	0.001-0.635	0.195-1.100	0.064-1.238	0.087-0.925
	variance	0.029	0.066	0.121	0.057
C. perfringens	n	8	8	6	12
	mean	0.480	0.622	0.502	0.596
	range	-0.043-0.954	0.173-0.866	0.184-1.007	0.337-0.966
	variance	0.088	0.057	0.088	0.048
C. sporogenes	n	8	8	6	12
	mean	0.771	0.996	0.696	0.819
	range	0.385-1.165	0.479-1.634	0.251-1.067	0.511-1.499
	variance	0.066	0.145	0.105	0.064

The representative data presented in Table 1 indicate that the overall range of ELISA OD values was broadly

similar for each of the three antigens. However, significant between-group variation was only evident for the C.novyi antigen.

The summarised clinical data provided in Table 1 support the assumptions that all Group I (AGS) and the majority of Group IV (miscellaneous clinical) horses have low levels of antibody to C. novyi and are therefore susceptible to EGS. Conversely horses in Group II (CGS) are expected to be variably seroconverted according to stage of 'incubation' at the time of sampling; inconclusive or positive results from the individual horses in the non-specific gastro-intestinal (Group III) animals may indicate involvement of a toxicoinfectious clostridial component in the disease process. Group I (AGS) and Group IV (miscellaneous clinical, non GE) may therefore be amalgamated to form a combined 'seronegative/inconclusive' category; similarly Group II (CGS) and III (clinical, GE) were statistically indistinguishable and may be combined to form an 'inconclusive/positive' category.

EXAMPLE 2

Comparison of these two combined groups I + IV and II + III provides a basis for meaningful analyses of variance.

TABLE 2

Analyses of variance of ELISA OD values for the three antigens based on amalgamated clinical grouping

	Seronegative/ inconclusive Groups I + IV	Inconclusive/ Seropositive Groups II + III	VR 1:34	Sig.
n	20	15		
C. novyi				
1:50	0.358	0.554	8.03	p<0.01
1:25	0.514	0.616	2.03	NS
C. perfringens				
1:50	0.550	0.553	0.00	NS
1:25	0.492	0.535	0.21	NS
C. sporogenes				
1:50	0.800	0.850	0.23	NS
1:25	0.852	0.816	0.09	NS

The individuality of the serological response to the three indicator antigens was further substantiated by the absence of significant correlation between the data sets.

TABLE 2A Correlation matrix for ELISA OD at 1:50 serum dilution

C.novyi	1.000		
C.perfringes	0.476 *	1.000	
C.sporogenes	0.429*	0.530**	1.000
	C.novyi	C.perfringens	C.sporogenes

EXAMPLE 3 ELISA of Clinical groups

(a) Group I Acute Grass Sickness (AGS)

Nine specimens were submitted from confirmed cases of AGS and with the exception of one specimen all were seronegative to the C.novyi antigen (i.e. OD <0.4). The single inconclusive result (OD 0.635) was recorded for a 12

year old horse in Group I which was also characterised by above group average seroconversion to the two non-specific antigens (Table 3).

TABLE 3

	ELISA OD Values		
AGS	N50	P50	S50
Horse 5			
(D20.91)	0.635	0.539	1.165
Group I min	0.001	0.043	0.385
mean	0.302	0.480	0.771
max	0.635	0.954	1.165
variance	0.029	0.088	0.066

(b) Group II Chronic Grass Sickness (CGS)

By definition horses susceptible to CGS are likely to be in the seronegative range (i.e. OD<0.4) at the outset of disease; clinical progression will be accompanied by a variable rate of seroconversion modified according to individual animal responses to the toxicoinfectious challenge. The majority of specimens included in Group II (confirmed CGS) were sampled at a relatively early stage in the disease process.

Mean duration at sampling 19 days

range 11-35 days

n = 23

(c) Group III Clinical GE

Table 4 provides a summary of the diagnoses or clinical signs recorded for the 12 horses included in

this category.

TABLE 4

Clinical GE - summary of diagnoses (or clinical signs) expressed in descending order of ELISA OD for C.novyi antigen at 1:50 serum dilution for Group III horses

	No.	N50 OD	Ref	Duration days	Diagnosis (signs)
Seropositive i.e. OD > 0.8	1	1.238	F456	LS	ND
	2	1.076	E50	LS	ND
	3	1.052	F462	LS	ND
	4	0.806	86/13	*	Pharyngeal paralysis
Inconclusive i.e. OD 0.4 - 0.8	5	0.744	E55	LS	ND
	6	0.718	926	*	Colitis X
	7	0.706	86/17	6.0	'colic'
	8	0.660	967	*	'diarrhoea'
	9	0.426	86.10	14.0	Pyloric stenosis
Sero-negative i.e. OD <0.4	10	0.350	92/64	1.0	Epiploic entrapment
	11	0.292	N1	*	Intussusception
	12	0.064	939	*	'diarrhoea'

Key
Duration - stage of disease at time of sampling
LS - long standing i.e. > 28 days
ND - not diagnosed
* - missing data

Nine horses were either positive (OD >0.8) or inconclusive (OD 0.4-0.8) to the ELISA N50 diagnostic test and were therefore serologically indistinguishable from the majority of chronic Grass Sickness cases (Group II). The remaining three listed horses were seronegative (OD < 0.4) and therefore indistinguishable from confirmed cases of acute Grass Sickness (Group I); it is noteworthy that cases

10 and 11 were acute abdominal crises and that the lowest OD value (0.064) was recorded from an 'undiagnosed' case of diarrhoea in a foal.

Horses 1, 2, 3 and 5 were all from endemic Grass Sickness areas and presented signs consistent with a 'field diagnosis of chronic Grass Sickness i.e. sporadic inappetance, mild colic and weight loss. However they could not be differentiated serologically from clinically normal animals in the same populations (Group V, mean OD 0.781).

Horse 6 (Colitis X) presented serological results which were indicative of C.perfringens involvement (OD 1.007) possibly associated with nonspecific seroconversion to the other two antigens (Table 5): conversely horse 4 (Pharyngeal paralysis) and horses 7 and 8 which were not diagnosed, did not show any evidence of non-specific seroconversion. The pyloric stenosis case (horse 9) had been affected 14 days prior to sampling; the N50 reaction was borderline (OD 0.426) but specific.

TABLE 5 Group III Pattern of responses to three indicator antigens in four clinical cases with gastro-enteric diagnoses/signs

Horse	Diagnosis/ 'signs'	N50	P50	S50
4	Pharyngeal paralysis	0.806	0.560	0.751
6	colitis X	0.718	1.007	0.875
7	'colic'	0.706	0.562	0.882
9	Pyloric stenosis	0.426	0.226	0.355
Overall OD	Minimum	0.001	0.043	0.251
	Mean	0.587	0.551	0.821
	Maximum	1.378	1.007	1.634
	(n)	(167)	(35)	(35)

Horses which present with undiagnosed gastro-enteric signs cannot be differentiated from early stage (i.e. <28 days duration) cases of chronic Grass Sickness. However in the absence of confirmed diagnosis it cannot be assumed that inconclusive C.novyi responses are non-specific. The data for horse 6 (colitis X) demonstrate that the test procedure can indicate the primary involvement of clostridia in non EGS disease processes; conversely serodiagnostic potential is limited by failure to differentiate possible cases of EGS (horses 1, 2 and 3) from normal 'immune' animals in endemic area populations.

(d) Group IV Clinical non GE

A total of 25 specimens were submitted from horses with various diagnosed 'non abdominal' disorders or presenting signs. In this category 15/25 i.e. 60% of specimens were seronegative to the C.novyi antigen at a

serum dilution of 1:50. The following table lists the data for the three positive horses (OD > 0.8) and for the seven inconclusive animals OD 0.4 - 0.8.

TABLE 6

Group IV horses : summary of diagnoses (or clinical signs) expressed in descending order of ELISA N50 OD for C.novyi antigen

	No.	N50 OD	Ref	Duration days	Diagnosis (signs)
Seropositive i.e. OD > 0.8	1	0.925	IN 3498	*	lame
	2	0.853	3611	*	ND
	3	0.831	4861	>728	lame
Inconclusive OD 0.4 - 0.8	4	0.754	C1	>28	weight loss
	5	0.698	IN 2995	*	ND
	6	0.639	86/27	>28	laminitis
	7	0.592	3479	*	lame
	8	0.516	A864	>28	'neurologic'
	9	0.475	IN 3297	>28	laminitis
	10	0.474	2302	*	ND

*Missing data

Four of the inclusive horses in the Group IV category were tested against the 'non specific' antigens and the results are presented in Table 7.

TABLE 7

Pattern of responses to three indicator antigens in miscellaneous clinical cases 'inconclusive' to the C.novyi antigen

Horse	Diagnosis (signs)	N50	P50	S50
5	ND	0.698	0.539	0.785
6	laminitis	0.639	0.912	1.499
9	laminitis	0.475	0.780	0.950
10	ND	0.474	0.966	0.745
Overall OD	Minimum	0.001	0.043	0.251
	Mean	0.587	0.551	0.821
	Maximum	1.378	1.007	1.634
	(n)	(167)	(35)	(35)

The two laminitis cases horses 6 and 9 both show evidence of non-specific seroconversion which is consistent with responses to concomitant enterotoxaemia.

EXAMPLE 3 - Diagnoses performed in normal horses.

Table 8 indicates the source and numbers of horses tested against the C.botulinum Group C indicator organism i.e. C.novyi. All routine tests were carried out at a 1:50 serum dilution.

TABLE 8

Categories of 'normal' horses tested*

	ENDEMIC AREAS EAST OF SCOTLAND	NON-ENDEMIC AREAS WEST OF SCOTLAND		
Group	V	VI	VII	VIII
Type	Mainly older horses	Horses in transit	High plane nutrition	Mixed grazing
Populations (n)	3	1	3	5

* *All groups contained one or more animals which were not clinically normal - mainly sporadic colic/diarrhoea or chronic 'weight loss'.*

Summarised data demonstrates the obvious between-group differentiation of the selected populations.

TABLE 9

ELISA OD values for 1:50 serum dilutions vs C.novyi antigen

Group	V	VI	VII	VIII
n	35	16	11	36
Mean	0.781	0.720	0.586	0.478
Range	0.313-1.287	0.462-1.378	0.261-1.026	0.179-1.081
Variance	0.060	0.071	0.056	0.064

The normal horses tested according to this Example are grouped as follows:

Group V

Horses in this category were 'lifetime' residents in endemic EGS areas of NE Scotland and included 20 animals grazing within two miles of the original Barry camp outbreak (1909); 33/35 Group V horses were seroconverted, irrespective of age.

Group VI

The majority of horses in Group IV were imported to Scotland from Ireland and were therefore likely to have been seronegative on arrival. At the time of sampling they had been 'in residence' for periods of one week to six months; the statistical pattern of seroconversion was indistinguishable from that of the Group V horses.

These limited data suggest that premises which are subject to 'regular traffic' are likely to become mini-endemic areas as a result of recurrent 'contamination', therefore highly susceptible newcomers may be 'at risk' especially when subjected to intercurrent nutritional or climatic stresses during the incubation period.

Groups VII and VIII

Eventers or race horses in 'full work' or training on a high plane of nutrition (Group VII) were included as a control category for the miscellaneous 'non-endemic' population (Group VIII).

Table 10 shows the pattern of seroconversion to the C.novyi antigen for the four non-clinical categories

TABLE 10

N50 OD	Groups V	VI	VII	VIII
Seronegative <0.4	2	0	1	13
Inconclusive 0.4-0.8	16	4	3	10
Seropositive >0.8	17	12	7	13
TOTAL	35	16	11	36

EXAMPLE 4

Therefore as a principle feature of the diagnostic procedure according to the invention, specimens from clinical cases which demonstrate inconclusive or positive serological responses to the indicator cell wall antigens are re-examined for the presence of "antitoxins" viz. antibodies to specific clostridial toxins such as the Clostridium botulinum Type C_1 neurotoxin. The results are presented in Tables 11 below:

TABLE 11

GROUP AI Acute EGS (n = 7)

I	II	III	IV	V
No./ Case ref.	Age in yrs	Cl novyi 1:50	ELISA OD's Cl botulinum (Cl antitoxin) 2×10^{-3}	4×10^{-3}
4	7.0	0.278	0.409	0.181
12	4.0	0.221	0.286	0.122
18	2.0	0.286	0.237	0.122
20	12.0	0.636	0.552	0.279
22	1.0	0.283	0.252	0.130
27	2.0	0.377	0.274	0.127
31	4.0	0.380	0.271	0.132
Group AII Chronic EGS (n = 8)				
2	9.0	0.335	0.172	0.087
5	5.0	0.378	0.250	0.123
24	4.0	0.698	0.425	0.208
25	12.0	0.890	0.432	0.195
26/ 90	8.0	0.391	0.214	0.009
26/ 91	9.0	0.465	0.238	0.129
28	3.0	0.639	0.153	0.097
34	4.0	0.954	0.206	0.096

TABLE 11 (Contd.)

Group BI

Clinical (Antitoxin ELISA OD > 0.10)

No./ Case ref.	Age in yrs	Diagnosis (signs)	Cl novyi	ELISA OD's Cl botulinum (Cl antitoxin) 2×10^{-3}	4×10^{-3}
35	19.0	Carcinoma	0.338	0.913	0.493
A	10.0	(Diarrhoea/ Colic)	1.221	0.312	0.182
13	*	(Pharyngeal paralysis)	0.806	0.274	0.142
64	10.0	Epiploic entrapment	0.350	0.196	0.091
B	7.0	(lame)	0.592	0.185	0.083
55	7.0	(trauma)	0.212	0.183	0.080
C	8.0	(lame)	0.925	0.140	0.065
77	12.0	Ovarian tumour	0.289	0.119	0.044

TABLE 11 (Contd.)

Group BII

Clinical (Antitoxin ELISA OD < 0.10)

No./ Case ref.	Age in yrs	* Diagnosis signs	Cl novyi	ELISA OD's Cl botulinum (Cl_3 antitoxin) 2×10^{-3}	4×10^{-3}
D	9.0	(respir-atory)	0.256	0.042	0.020
E	12.0	Pyelone-phritis	0.371	0.041	0.020
F	11.0	Colitis X	0.718	0.034	0.010
G	14.0	*	0.698	0.033	0.016
H	15.0	Laminitis	0.475	0.030	0.001
J	*	(lame)	0.087	0.022	0.027
K	0.5	(diarrhoea)	0.650	0.013	0.006

TABLE 11 (Contd.)

GROUP C

Seroconversion vs duration of clinical signs (days)

CI 0.5 - 3.0 days

No.	Ref	Duration (days)	ELISA OD (*Clostridium novyi*) 1:50
1	E4	0.5	0.278
2	E22	0.5	0.283
3	E31	0.5	0.380
4	E27	1.5	0.377
5	E18	2.0	0.286
6	E12	3.0	0.221
CII 10.0 - 15.0 days			
1	N2	11.0	0.649
2	N8	11.0	0.437
3	E2	12.0	0.335
4	26/91	13.0	0.465
5	N6	14.0	0.358
6	N10	14.0	0.938
7	E24	15.0	0.698

TABLE 11 (Contd.)

CIII > 15 days			
1	E28	16.0	0.639
2	N3	16.0	0.893
3	E25	18.0	0.890
4	N4	21.0	0.770
5	N13	21.0	0.727
6	N12	27.0	1.092
7	E34	33.0	0.954

EXAMPLE 5

Demonstration of Clostridium botulinum Type C_1 neurotoxin in the intestinal content of horses affected with EGS.

Faecal specimens were collected at autopsy in a series of horses affected with EGS; in all cases the diagnosis was confirmed prior to euthanasia by the presence of characteristic neuronal lesions in ileal biopsy specimens.

Approximately 20 gram specimens of faeces were transferred to 20 ml volumes of phosphate buffered saline pH 7.2 containing 0.2% gelatin (PBSG) to preserve the toxin; the PBSG samples were mixed to disperse the specimen and then allowed to infuse at 4°C for 24 hours prior to long-term storage at -30°C.

The presence and potency of botulinum C_1 neurotoxin was determined and assayed in the supernatant fluid of PBSG infusions by a modification of the ELISA protocol which involves precoating the reaction plates with a standardised C. botulinum Type C_1 antitoxin.

Method

The use of sandwich ELISA for the detection of botulinum Type C_1 neurotoxin.

1. An ELISA plate was coated (100 µl well) with guinea pig antibody specific for Clostridium botulinum type C neurotoxin at 5-10 µg/ml using phosphate buffered saline (PBS) as a diluent. The antibody solution was added and the plate was shaken for five minutes before incubation

overnight at 4°C.

2. The plate was washed once using PBS containing 0.1% Tween 20 (PBS-T).

3. The blanking solution of PBS-T containing 5% foetal bovine serum (FBS) was added (100 µl/well) and the plate was incubated for 1 hour at 37°C with continuous shaking. Unbound materials were removed by means of washing as above.

4. The antigen solution prepared in PBS-T containing 5% FBS (10 µg/ml; 1:5 dilutions) was added (100 µl/well). Incubation was carried out in plate for 60-90 minutes at 37°C with continuous shaking.

5. The plate was washed three times using PBS-T.

6. The second antibody-enzyme conjugate (100 µl/well of guinea pig antibody labelled with horse radish peroxidase) prepared in PBS-T containing 5% FBS was added. Incubation of the plate was carried out for 60-90 minutes at 37°C with continuous shaking.

7. The plate was washed three times using PBS-T.

8. The substrate* solution (TMB) was added and the enzyme was allowed to react.

9. The reaction was stopped with 50 µl/well of H_2SO_4, 2M before measuring the absorbance at 450mm.

* 10 mg TMB in 1 ml DMSO was dissolved in a dark glass tube. 100 µl of the TMB solution was added to 10 ml phosphate/citrate buffer, pH 5.0 containing 44 µl 1% H_2O_2.

The results are shown below in Table 12.

TABLE 12 Detection and assay of botulinum C_1 neurotoxin in the intestinal content of horses affected with EGS.

Case Ref:	ELISA OD VALUES		Botulinum C_1 toxin* (ng/ml)
	Serum C.novyi antibody	PBSG extract botulinum C1 toxin	
733	0.798	1.017	8.8
851	NS	0.729	6.3
869	0.897	NS	-
889	0.685	1.305	11.4

NS not sampled

* 1.0 ng botulinum C_1 toxin represents approximately 100 mouse MLD'S.

The demonstration of lethal concentrations of botulinum C_1 neurotoxin in faecal infusions derived from 3/4 horses affected with EGS, combined with concomitant serological evidence of exposure to a phenotype of C. novyi further attests to the toxicoinfectious nature of the disease process. The evidence also suggests that the contagious source of the C_1 neurotoxin is located in the tissues, or lumen, of the bowel.

EXAMPLE 6

Confirmation of the location of a botulinum C_1 toxin-producing organism in the tissues of horses affected with EGS.

The neuronal lesions of EGS invariably affect the ileum which is therefore likely to be a predilection site for the establishment of toxicoinfectious contagion.

Approximately 20 gram samples of ileum and/or spleen tissues were taken at autopsy of five confirmed cases of EGS and transferred immediately to 50 ml volumes of prereduced enrichment culture media. The selected medium (designated AIM/CMB/SSG) was a modified version of cooked meat broth fortified with glucose (0.2%) and soluble starch (0.3%) to optimise conditions for primary isolation of fastidious anaerobes and also incorporated gentamycin (10 ml/l) to inhibit the overgrowth of dominant or contaminant species. The sample bottles were incubated anaerobically for 5 days at 30°C; supernatant fluids were then harvested and tested for the presence of toxin by the ELISA technique as used in the preceding example.

The results are shown in the following table:

TABLE 13

Isolation of botulinum C_1 neurotoxin-producing C. botulinum from the tissues of horses affected with EGS.

Case Ref.	Sample type	C. botulinum ELISA OD*	Type C_1 neurotoxin ng/ml
733	Faeces^{+ve}		
	Ileum - isolate pending identification		
	Spleen - no toxin detected		
830	Spleen	1.424	12.4
851	Faeces^{+ve}		
	Ileum	1.312	57.0**
869	Faeces^{-ve}		
	Ileum	1.449	12.6
	Spleen - no toxin detected		
889	Faeces^{+ve}		
	Ileum - no toxin detected		
	Spleen - no toxin detected		

Sample dilution * 1/5

** 1/25

The demonstration of botulinum C_1 neurotoxin in 3/5 AIM/CMB/SSG broth fluid supernates confirms the presence therein of a viable serotype of C. botulinum type C or D. A combination of data presented in Examples 5 and 6 above indicates that the immunoassays successfully demonstrate the presence of lethal concentrations of botulinum C_1 neurotoxin in faecal fluids and/or in tissue culture

supernates derived from five successive confirmed cases of EGS. These positive associations of a potent toxin and the parent toxinogenic anaerobe with clinical EGS confirms the diagnostic validity of the differential serological test here presented as the inventive procedure.

CLAIMS

1. A method for the diagnosis of equine toxicoinfectious clostridiosis which comprises detecting the presence of an antibody to a C. botulinum antigen or a phenotype thereof and/or an antibody to a clostridial toxin in a biological sample.

2. A method according to claim 1, for the diagnosis of equine grass sickness which comprises detecting the presence of an antibody to C. botulinum Group III antigen or a phenotype thereof and an antibody to a botulinum type C toxin.

3. A method according to any of claims 1 or 2, wherein the phenotype is derived from C. novyi or C. perfringens.

4. A method according to claim 3 for the detection of anterior enteritis or colitis X, wherein the phenotype is derived from C. perfringens.

5. A kit for diagnosis of equine toxicoinfectious clostridiosis which comprises means for detecting an antibody to a C. botulinum antigen or a phenotype thereof, and/or means for detecting an antibody to a clostridial toxin.

6. A kit according to claim 5 for diagnosis of equine grass sickness, which comprises means for detecting the presence of an antibody to C. botulinum Group III antigen or a phenotype thereof and means for detecting an antibody to a botulinum type C toxin.

INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOLOGICAL ABSTRACTS, vol. 65, no. 12, 15 June 1978 Philadelphia, PA, US; abstract no. 71495, T. ITOH ET AL.: "An outbreak of botulism on a mink farm in Hokkaido." page 7032; XP002006001 see abstract & BULL. AZABU. VET. COLL., vol. 1, no. 2, 1976, pages 29-35, --- -/--	1,2,5,6

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Cartagena y Abella,P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AMERICAN JOURNAL OF VETERINARY RESEARCH, vol. 39, no. 6, 1978, pages 1049-1051, XP000573469 R. OCHOA ET AL.: "Equine Grass Sickness: serologic evidence of association with Clostridium perfringens Type A enterotoxin." ---	
A	EQUINE VETERINARY JOURNAL, vol. 13, no. 1, 1981, pages 56-58, XP000573433 J.S. GILMOUR ET AL. : "A negative serological relationship between cases of grass sickness in Scotland and Clostridium perfringens type A enterotoxin." ---	
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A	ONDERSTEPOORT JOURNAL VETERINARY RESEARCH, vol. 46, no. 3, 1979, pages 121-124, XP000571955 R. W. WORTHINGTON ET AL.: "Isolation and characterization of antibodies to Clostridium perfringens epsilon toxin from hyperimmune horse serum." ---	
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